REMARKS

Upon entry of this amendment, claims 14, 16, 17, 20, 22, and 24 are currently pending in this application. Claims 1-8 were previously canceled and claims 9-13, 15, 18, 19, 21, and 23 are canceled herein, all without prejudice or disclaimer. Claims 14, 17, 20, 22, and 24 are amended herein. Support for those amendments can be found throughout the specification, *e.g.*, in original claims 2, 4, and 8; the Title and Abstract; page 3, lines 5-7; page 7, lines 21-33; and the Examples. The Brief Description of the Drawings section of the specification is amended to recite "activated Factor XIII," where appropriate. Support for those amendments can be found throughout the specification, *e.g.*, in the Examples. The specification is also amended to capitalize the trademarks Matrigel® and Affi-Gel® and provide appropriate generic terminology. Support for the generic terminology can be found, *e.g.*, in the Matrigel® and Affi-Gel® product sheets submitted herewith. Thus, no new matter has been added by these amendments.

WITHDRAWN REJECTIONS

Applicant acknowledges, with appreciation, that the Office has withdrawn the finality of the Office Action mailed October 2, 2008, and the rejection of claims 9-24 under 35 U.S.C. § 102(b) as allegedly anticipated by WO 98/51333 to Deisher et al. ("*Deisher*").

OBJECTIONS TO THE SPECIFICATION

The Office objects to the specification because (1) "[t]he use of the trademarks, e.g., Matrigel, Affigel, etc., has been noted in this application. It should be capitalized

wherever it appears and be accompanied by the generic terminology"; and (2) "the term [activated Factor XIII] should be applied properly to the descriptions of the drawings." (Office Action at p. 2.)

The specification is amended to capitalize the trademarks Matrigel® and Affi-Gel® and provide appropriate generic terminology. In addition, the Brief Description of the Drawings section of the specification is amended to recite "activated Factor XIII," where appropriate. Accordingly, Applicant respectfully requests that the Office withdraw these objections to the specification.

REJECTION UNDER 35 U.S.C.§ 103(a)

Claims 9-24 are rejected under 35 U.S.C. § 103(a) as allegedly obvious over *Deisher* in view of Dardik R. et al., "Novel Proangiogenic Effect of Factro XIII Associated With Suppression of Thrombospondin 1 Expression," Arterioscler Thromb Vasc Biol., 23:1472-77 (2003) ("*Dardik*") and US 2003/0228371 to Skinner et al. ("*Skinner*"). (Office Action at pp. 2-7.) Specifically, the Office acknowledges that *Deisher* fails to "teach administration of Factor XIIIa" (*id.* at p. 4) but contends that *Dardik* and *Skinner* cure these deficiencies in *Deisher* because *Dardik* "teach that [activated] factor XIII participates in tissue remodeling and wound healing, and processes that involve angiogenesis" (*id.* at p. 6) and *Skinner* "teach the use of molecules related to Factor

XIIIa" (*id.* at p. 5)[1] to "reduce the effects of ischemic events, such as infarctions caused by reperfusion and/or oxygen deprivation" (*id.* at p. 6). Applicant respectfully traverses.

Several basic factual inquiries must be made to determine whether the claims of a patent application are obvious under 35 U.S.C. § 103. These factual inquiries, set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966), require the Examiner to:

> (1) Determine the scope and content of the prior art;
>
> (2) Ascertain the differences between the prior art and the claims in issue;
>
> (3) Resolve the level of ordinary skill in the pertinent art; and
>
> (4) Evaluate evidence of secondary considerations.

The obviousness or non-obviousness of the claimed invention is then evaluated in view of the results of these inquiries. *Graham*, 383 U.S. at 17-18; *see also KSR Int'l Co. v. Teleflex, Inc.*, 127 S. Ct. 1727, 1734 (2007). "To reach a proper determination under 35 U.S.C. § 103, the Examiner must step backward in time and into the shoes worn by the hypothetical 'person of ordinary skill in the art' when the invention was unknown and just before it was made." M.P.E.P. § 2142, 8th Ed., July 2008 Rev. Once the findings of fact are articulated, the Office "must then make a determination whether the claimed invention 'as a whole' would have been obvious at the time to that person." *Id.*

Based on the Supreme Court's decision in *KSR*, the Patent Office has announced seven exemplary rationales that may support a conclusion of obviousness.

---

[1] Applicant respectfully disagrees that the Fibrinogen A (FPA) molecule disclosed in *Skinner* is "related to Factor XIIIa," as alleged by the Office. In fact, *Skinner* indicates that FPA and Factor XIII play non-analogous roles in the fibrinogen cascade (*id.* at [0129]-[0133]) and thus, provides no reason to expect that the two proteins are similar. Accordingly, Applicant respectfully submits that the Office's comparison of FPA and Factor XIII is improper in the absence of evidence demonstrating that the molecules share structural and functional similarity.

See M.P.E.P. § 2143. All of these bases for obviousness require that one of ordinary skill in the art, without knowing anything of the claimed invention, would not only be motivated to produce that invention, but also would have a reasonable expectation of success and achieve predictable results. Applicant respectfully submits that the Office has failed to establish a *prima facie* case of obviousness because one skilled in the art would not have predicted, based on the cited references, that activated Factor XIII will stimulate the perfusion of ischemic tissues by inducing the proliferation of new blood vessels, as required by the currently pending claims.

The Office cites *Dardik* for disclosing that activated Factor XIII has proangiogenic effects in an in vitro rabbit cornea model. (*See* Office Action at p. 6.) The Office acknowledges that *Dardik* "does not specifically teach the treatment [of Factor XIII] for stimulating the perfusion of ischemic tissues." (*Id.*) However, the Office contends that it would have been obvious to use Factor XIII to stimulate perfusion of ischemic tissues by inducing the proliferation of new blood vessels, since *Deisher* discloses treating ischemic tissue with Factor XIII and allegedly "addresses all diseases which are associated with disturbed blood perfusion and thus include instant claims that refer to stimulating the perfusion of ischemic tissues" (Office Action at p. 4) and *Skinner* discloses that a component of the fibrinogen cascade, Fibrinogen A, allegedly has "anti-infarction, i.e., increased perfusion ... activity" (Office Action at p. 7). Applicant respectfully disagrees.

Applicant acknowledges that *Dardik* discloses "in an in vitro model, FXIIIa significantly induces new vessel formation in a rabbit cornea" and that "[t]he proangiogenic effect of FXIIIa is associated with downregulation of thrombospondin

(TSP-1) ... ." (Specification at p. 2, ll. 32-22.) However, *Dardik* also reports that these observations "contradict the data reported by Dallabrida et al, who showed that FXIIIa inhibits capillary tube formation by human microvascular endothelial cells in a fibrin gel," and suggests that this discrepancy "may indicate that FXIIIa may have variable effects on different types or sources of endothelial cells." *Dardik* at p. 1477, 2nd col. For example, "if the proangiogenic effect of FXIIIa is mediated at least in part by TSP-1, it is possible that cells with lower TSP-1 synthesis will be less responsive to the effect of FXIIIa." *Id.* Thus, based on the teachings of *Dardik*, one skilled in the art would not predict that the proangiogenic effect of FXIIIa observed in rabbit corneas would also occur in ischemic tissues, or that activated Factor XIII could be used to stimulate perfusion of ischemic tissues, as required by the currently pending claims.

Neither *Deisher* nor *Skinner* dispel the doubts raised in *Dardik* regarding the applicability of Factor XIII for inducing blood vessel proliferation in ischemic tissues, since these references do not teach or suggest any role for Factor XIII in stimulating the proliferation of new blood vessels in ischemic tissues. Indeed, *Deisher* does not discuss angiogenesis at all, and although *Skinner* makes a passing reference to "methods utilizing animal models of new vessel angiogenesis" (*id.* at [0082] and [0127]), *Skinner* provides no data to establish any connection between Fibrinogen A and new vessel angiogenesis. In fact, *Skinner* teaches that "[t]reatment of ischemia typically involves reduction of blockage" (*id.* at [0196]), which essentially teaches away from treating ischemia by inducing the proliferation of new blood vessels, as required by the currently pending claims.

Moreover, *Deisher* and *Skinner* fail to teach or suggest that Factor XIII plays any role in stimulating the perfusion of ischemic tissues. Applicant acknowledges that both references teach methods for reducing the negative side-effects of ischemic events. Specifically, *Skinner* discloses anti-infarction molecules that can be used to "reduce the effects of ischemic events, such as infarctions caused by reperfusion and/or oxygen deprivation" (*Skinner* at [0195]) and *Deisher* teaches methods for reducing "tissue damage or vascular injury following restoration of blood flow (reperfusion)" (*Deisher* at p. 9, ll. 28-29). However, methods for reducing the negative side-effects of ischemic events are not equivalent to methods for treating ischemic tissues comprising stimulating the perfusion of ischemic tissues by inducing the proliferation of new blood vessels, as required by the currently pending claims. Indeed, *Deisher* and *Skinner* are notably silent regarding any role for Factor XIII or Fibrinogen A in stimulating the perfusion of ischemic tissues, and instead describe physiological mechanisms influenced by Factor XIII that are different and independent from the instant invention. Accordingly, Applicant respectfully submits that the Office has failed to provide an articulated reasoning with some rational underpinning for the statements that the anti-infarction molecules disclosed in *Skinner* "increase perfusion" (Office Action at p. 7) or that *Deisher* "addresses all diseases which are associated with disturbed blood perfusion" (*id.* at p. 4). In fact, based on the teachings of *Deisher* and *Skinner* disclosing the use of Factor XIII or Fibrinogen A, respectively, for reducing the negative side-effects of ischemic events, one skilled in the art would not predict that activated Factor XIII can be used to stimulate perfusion of ischemic tissue, and certainly would

not predict that Factor XIII stimulates the proliferation of new blood vessels, as required by the currently pending claims.

At the time of the invention, "[w]hether Factor XIII participates in angiogenesis in ischemic tissue [was] not definitely known." (Specification at p. 2, ll. 28-29.) In fact, those skilled in the art at the time expressed significant doubt regarding whether activated Factor XIII would exhibit proangiogenic properties in tissues other than rabbit cornea. *See Dardik* at p. 1477, 2nd col. Applicant has demonstrated for the first time that activated Factor XIII stimulates the perfusion of ischemic tissues by inducing the proliferation of new blood vessels. (*See* Example 3.) These unexpected results are recited in the currently pending claims. When a patent application claims a combination of prior art elements that work together in an unexpected and fruitful manner, the invention is non-obvious. *See KSR*, 127 S. Ct. at 1740 (2007). Thus, the demonstrated operability of the invention, despite the skepticism of those skilled in the art, establishes the nonobviousness of the instantly claimed invention.

For at least these reasons, Applicant respectfully requests that the Office withdrawn this rejection under 35 U.S.C. § 103(a).

CONCLUSION

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration and reexamination of this application and the timely allowance of the pending claims.

Please grant any extensions of time required to enter this response and charge any additional required fees to Deposit Account No. 06-0916.

Respectfully submitted,

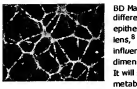
FINNEGAN, HENDERSON, FARABOW,
GARRETT & DUNNER, L.L.P.

Dated: June 23, 2009

By: Amanda K. Murphy
Amanda K. Murphy
Reg. No. 59,387

BD Matrigel™ Basement Membrane Matrix

Introduction Product Info



Buy now »

BD Matrigel™ Basement Membrane Matrix is effective for the attachment and differentiation of both normal and transformed anchorage dependent epithelioid and other cell types. These include neurons,[5,6] Sertoli cells,[7] chick lens,[8] and vascular endothelial cells,[9] and hepatocytes.[10] BD Matrigel will influence gene expression in adult rat hepatocytes[11] as well as three dimensional culture in mouse[12-15] and human[16,17] mammary epithelial cells. It will support *in vivo* peripheral nerve regeneration,[18-20] can be used for metabolism and toxicology studies,[21,22] and is the basis for several types of tumor cell invasion assays.[23,24] BD Matrigel provides the substrate necessary for the study of angiogenesis both *in vitro* [25,26] and *in vivo*.[27-29] BD Matrigel also supports *in vivo* propagation of human tumors in immunosupressed mice.[30-32]

Features

- Certified LDEV-Free
- Extensive quality control exceeding industry standard
- Rigorously validated for functionality
- Most extensively referenced basement membrane matrix

Wide Selection of Basement Membranes

- **BD Matrigel Matrix Growth Factor Reduced** (GFR) is suited for applications where a more highly defined basement membrane preparation is desired. Available in Standard GFR, High Concentration, and Phenol-Red Free formats.
- **BD Matrigel Matrix High Concentration** (HC) is suited for *in vivo* applications where a high protein concentration augments growth of tumors. The high protein concentration also allows the BD Matrigel Matrix Plug to maintain its integrity after subcutaneous injection into mice. Available in Standard, Growth Factor Reduced (GFR) and Phend-Red Free formats.
- **BD Matrigel Matrix Phenol-Red Free** format is recommended for assays which require color detection (i.e. fluorescence).
- **BD Matrigel hESC-Qualified Matrix** has been qualified as mTeSR™1E®-compatible by StemCell Technologies, thus eliminating the need for time-consuming screening, in order to provide the reproducibility and consistency essential for your human embryonic stem (hES) cell research. The mTeSR™1 formulation and BD Matrigel Matrix have been shown to be a successful combination for feeder-free maintenance of different WiCell™ hES cell lines for up to 20 passages. (mTeSR™1 Cat. No. 05850.)

Applications

Cell Growth and Differentiation
BD Matrigel Matrix is especially suited for the culture of polarized cells, such as epithelial cells. It promotes the differentiation of many cells types, including hepatocytes, mammary epithelial, endothelial, smooth muscle cells and neurons.

The acini shown were stained with H/E at 72 hours. Indirect immunofluorescence staining also revealed the salivary gland specific cysteine protease inhibitor, cystatin, in HSG cell acini (data not shown). (Photo courtesy of Dr. Hynda Kleinman).

Metabolism/Toxicology Studies
BD Matrigel Matrix has been used to successfully construct *in vitro* models of liver cells for drug toxicity studies.

Note the clusters of spherical cells for hepatocytes cultured on BD Matrigel Matrix, typical of differentiated cells.

Invasion Assays
BD Matrigel Matrix provides a biologically active basement membrane model for in vitro invasion assay.

Scanning electron micrograph of two human fibrosarcoma cells, having digested the BD Matrigel Matrix occluding the membrane and migrating through the 8 Åµm of the PET membrane.

***In Vitro* and *In Vivo* Angiogenesis Assays**
BD Matrigel Matrix serves as a substrate for *in vitro* endothelial cell invasion and tube formation assays. It can also be used to assess *in vivo*

Related Products

- BD BioCoat™ Cellware
- BD BioCoat™ Matrigel™ Matrix Cell Culture Inserts
- BD BioCoat™ Matrigel™ Invasion Chambers
- BD BioCoat™ Tumor Invasion System
- BD BioCoat™ Angiogenesis Systems Dispase
- BD Cell Recovery Solution

angiogenic activity of different compounds via the BD Matrigel Plug Assay.

***In Vivo* Angiogenesis Studies and Augmentation of Tumors in Immunosuppressed Mice**

BD Matrigel Matrix High Concentration is suited for *in vivo* applications where a high protein concentration augments growth of tumors. The high protein concentration also allows the BD Matrigel Matrix Plug to maintain its integrity after subcutaneous injection into mice. This keeps the injected tumor cells and/or angiogenic compounds localized for in situ analysis and/or future excision. Available in standard Growth Factor Reduced (GFR) and Phenol-Red Free formats.

Characterization

Average Growth Factor Comparison of BD Matrigel

Parameter	BD Matrigel Matrix	BD Matrigel Matrix Growth Factor Reduced
bFGF (pg/ml)	0 - 0.1	0 - 0.1
EGF (ng/ml)	0.5 - 1.3	< 0.5
IGF-1 (ng/ml)	15.6	5
PDGF (pg/ml)	12	< 5
NGF (ng/ml)	< 0.2	< 0.2
TGF-beta (ng/ml)	2.3	1.7
% Protein that gels	80	83

ECM Composition of BD Matrigel Matrix vs. GFR Matrigel Matrix

Matrigel Component	Percent in BD Matrigel Matrix	Percent in BD Matrigel Matrix Growth Factor Reduced
Laminin	56%	61%
Collagen IV	31%	30%
Entactin	8%	7%

Quality Control

- Mouse colonies are routinely screened for pathogens via Mouse Antibody Production (MAP) testing
- Extensive PCR testing is performed on a number of pathogens, including LDEV, to ensure strict control of raw materials used during the manufacturing of BD Matrigel
- Tested and found negative for bacteria, fungi, and mycoplasma
- Protein concentrations are determined by Lowry method
- Endotoxin units are measured by Limulus Amoebocyte Lysate assay
- BD Matrigel gel stability is tested for a period of 14 days at 37ºC

Biological activity is determined for each lot using a neurite outgrowth assay. Chick dorsal root ganglia are plated on a 1.0 mm layer of BD Matrigel Matrix and must generate positive neurite outgrowth response after 48 hours without addition of nerve growth factor

Technical Documents

TB No.	Title/Author
435	Methods for Implantation of BD Matrigel™ Matrix into Mice and Tissue Fixation *Kazuo Ohashi, M.D., Ph.D., Takashi Yokoyama, M.D., Yoshiyuki Nakajima, M.D., Ph.D.,* and *Marshall Kosovsky, Ph.D.*

References

Introduction

1. Kleinman, H.K., et al., Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma, Biochemistry, 21:6188 (1982).

2. Kleinman, H.K., et al., Basement membrane complexes with biological activity, Biochemistry, **25**:312 (1986).
3. Vukicevic, S., et al., Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular activity related to extracellular matrix components, Experimental Cell Research, **202**:1 (1992).
4. McGuire, P.G. and Seeds, N.W., The interaction of plasminogen activator with a reconstituted basement membrane matrix and extracellular macromolecules produced by cultured epithelial cells, J. Cell. Biochem., **40**:215(1989).

Cell Growth and Differentiation

5. Biederer, T. and Scheiffele, P.Â Mixed-culture assays for analyzing neuronal synapse formation, Nature Protocols, **2**(3):670 (2007).
6. Li, Y., et al., Essential Role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor, Nature, **434**:894 (2005).
7. Hadley, M.A., et al., Extracellular matrix regulates sertoli cell differentiation, testicular cord formation, and germ cell development in vitro, J. Cell Biol., **101**:1511 (1985).
8. Ireland, M.E., Quantification and regulation of mRNAs encoding beaded filament proteins in the chick lens, **16**(8):838 (1997).
9. McGuire, P.G., and Orkin, R.W., A simple procedure to culture and passage endothelial cells from large vessels of small animals, Biotechniques, **5**(6):456 (1987).
10. Bissell, D.M., et al., Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver, J. Clinical Invest., **79**:801 (1987).
11. Page, J.L., et al., Gene expression profiling of extracellular matrix as an effector of human hepatocyte phenotype in primary cell culture, Toxilogical Sciences, **97**(2):384 (2007).
12. Li, M.L., et al., Influence of a reconstituted basement membrane and its components on casein gene expression and secretion in mouse mammary epithelial cells, Proc. Nat. Acad. Sci. USA, **84**:136 (1987).
13. Barcellof, M.H., et al., Functional differentiation and aveolar morphogenesis of primary mammary cultures on reconstituted basement membrane, Development, **105**:223 (1989).
14. Roskelley, C.D., et al., Extracellular matrix-dependent tissue-specific gene expression in mammary epithelial cells requires both physical and biochemical signal transduction, Proc. Nat. Acad. Sci. USA, **91**(26):12378 (1994).
15. Xu, R., et al., Extracellular matrix-regulated gene expression requires cooperation of SWI/SNF and transcription factors, J. Biol. Chem., **282**(20):14992(2007).
16. Debnath, J., et al., Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures, Methods, **30**(3):256 (2003).
17. Muthuswamy, S.K., et al., ErbB2, but not ErbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini, Nat. Cell Biol., **3**(9):785 (2001).
18. Madison, R., et al., Increased rate of peripheral nerve regeneration using bioresorbable nerve guides and laminin containing gel, Exp. Neurology, **88**:767 (1985).
19. Xu, X.M., et al., Axonal regeneration into Schwann cell-seeded guidance channels grafted into transected adult rat spinal cord, J. Comp. Neurol., **351**(1):145 (1994).
20. Fouad, K., et al., Combining schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase promotes locomotor recovery after complete transection of the spinal cord, The Journal of Neuroscience, **25**(5):1169 (2005).

Metabolism/Toxicology Studies

21. Bi, Y., et al., Use of cryopreserved human hepatocytes in sandwich culture to measure hepatobiliary transport, Drug Metabo. and Dispos., **34**(9):1658 (2006).
22. Yu, X., et al., Essential role of extracellular matrix (ECM) overlay in establishing the functional integrity of primary neonatal rat sertoli cell/gonocyte co-cultures: An improved in vitro model for assessment of male reproductive toxicity, Toxilogical Sciences, **84**(2):378 (2005).

Invasion Assays

23. Terranova, V.P., et al., Use of a reconstituted basement membrane to measure cell invasiveness and select for highly invasive tumor cells, Proc. Nat. Acad. Sci. USA, **83**:465 (1986).
24. Albini, A., et al., A rapid in vitro assay for quantitating the invasive potential of tumor cells, Cancer Research, **47**:3239 (1987).

In Vitro Angiogenesis Assay

25. Kubota, Y., et al., Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures, J. Cell Biol., **107**:1589 (1988).
26. Maeshima, Y., et al., Identification of the anti-angiogenic site within vascular basement membrane-derived Tumstatin, J. Biol. Chem., **276**(18):15240(2001).

In Vivo Angiogenesis Assays and Augmentation of Tumors in Immunosuppressed Mice

27. Passaniti, A., et al., A simple, quantitative method for assessing angiogenesis and anti-angiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor, Lab Invest., **67**:519 (1992).
28. Iaeji, M., et al., Tranilast inhibits the proliferation, chemotaxis and tube formation of human microvascular endothelial cells in vitro and angiogenesis *in vivo*, British Journal of Pharmacology, **122**:1061 (1997).
29. Kisucka, J., et al., Platelets and platelet adhesion support angiogenesis while preventing excessive hemorrhage, Proc. Nat. Acad. Sci. USA, **103**(4):855 (2006).
30. Albini, A., et al., Matrigel promotes retinoblastoma cell growth in vitro and *in vivo*, Int. J. Cancer, **52** (2):234 (1992).
31. Yue, W., et al., MCF-7 human breast carcinomas in nude mice as a model for evaluating aromatase inhibitors, J. Steroid Biochem. Molec. Biol., **44**(4-6):671 (1993).
32. Angelucci, A., et al., Suppression of EGF-R signaling reduces the incidence of prostate cancer metastasis in nude mice, Endocrine-Related Cancer, 13(1):197 (2006).

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High Efficiency Coupling for All Primary Amino Group Ligands

Affi-Gel 10 and Affi-Gel 15 are complementary affinity media that offer rapid, high-efficiency coupling for all ligands that have a primary amino group — including proteins with any isoelectric point (pI) and low molecular weight ligands. Both Affi-Gel 10 and 15 are N-hydroxysuccinimide esters of a derivatized crosslinked agarose gel bead support (Figure 1), and both couple to ligands spontaneously in aqueous or nonaqueous solution.

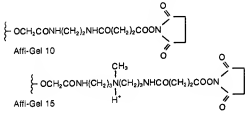

Fig. 1. Chemical structures of Affi-Gel 10 and Affi-Gel 15.

Affi-Gel 10, which contains a neutral 10-atom spacer arm, has been used to couple a variety of materials in affinity chromatography, immunoadsorption, and other techniques (see applications sections below). Affi-Gel 15 contains a positive charge in its 15-atom spacer arm, which significantly enhances coupling efficiency for acidic proteins at physiological pH. Both Affi-Gel 10 and Affi-Gel 15 offer the following additional advantages:

High Stability

Ether bonds link the spacer arm to chemically crosslinked agarose gel beads (Bio-Gel® A-5m support). Amide bonds couple the protein ligand to the terminal carboxyl of the spacer arm. Excellent resistance to urea, guanidine HCl, heat, solvents, acid, and base (pH 2–11) virtually eliminates ligand leakage during storage and use (Conn et al. 1981, La Porte et al. 1977).

Rapid, Gentle Coupling

Proteins are coupled with high efficiency within 4 hr at 4°C. The N-hydroxysuccinimide active ester method is highly selective for primary amino groups of ligands under physiological conditions.

Easy to Use

Wash Affi-Gel support with cold deionized water, add to buffered protein solution, and gently agitate. Low molecular weight ligands can be coupled in either aqueous or anhydrous solutions. Affi-Gel 10 and Affi-Gel 15 are supplied fully swelled and solvent-stabilized in isopropyl alcohol. The active ester content of both media is 15 µmol/ml.

Coupling Chemistry

Ligands with free alkyl or aryl amino groups will couple spontaneously with Affi-Gel 10 or 15 in aqueous or nonaqueous solution (Figure 2). Upon addition of ligand, the N-hydroxysuccinimide is displaced and a stable amide bond is formed. Since the reactive ester is immobilized on the gel and is highly selective for primary amino groups, spurious side reactions with the ligand (i.e., crosslinking or other modification in free solution) are eliminated. Among functional groups other than primary amines, free sulfhydryls are known to complete for coupling. In aqueous solution at neutral pH, Affi-Gel 10 will undergo gradual hydrolysis to yield a terminal carboxyl group.

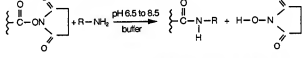

Fig. 2. Coupling reaction of Affi-Gel with ligand containing free amino groups.

Affi-Gel 10 and Affi-Gel 15 are well suited for coupling low molecular weight ligands. This can be done in aqueous solution or, when solubility of the liquid permits, in organic solvent.

Aqueous Coupling Conditions

pH

A major advantage of Affi-Gel 10 and 15 is the range of mild conditions that will permit coupling. This is particularly advantageous in applications that involve sensitive enzymes or other proteins that irreversibly lose biological activity when exposed to conditions outside of their physiological range. Useful coupling can be achieved with Affi-Gel 10 and 15 in the pH range of 3.0–10.0.

In order to maintain pH control, a minimum buffer strength of 0.01 M is recommended. Suitable buffers include MES, MOPS, HEPES, POPSO, acetate, and bicarbonate. Buffers such as Tris or glycine, which contain primary amino groups, will couple to the gel, as will any primary amine-containing compound that contaminates the ligand preparation.

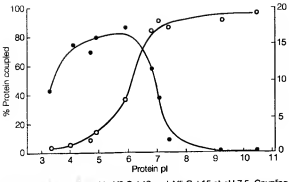

Fig. 3. Protein coupling with Affi-Gel 10 and Affi-Gel 15 at pH 7.5. Coupling conditions: Each protein solution (40 mg protein in 3 ml of 0.1 M MOPS, pH 7.5) was combined with 2 ml of Affi-Gel. The gel slurry was mixed at 4°C for 2 hr, then stripped with 7 M urea containing 1 M NaCl. The uncoupled protein was determined using published $E_{2\%}^{1cm}$ (Twining and Brecher 1975) by dilution of an aliquot of the urea effluent into 0.1 N HCl and measurement of the absorbance at 280 nm (○—○, Affi-Gel 10; ●—●, Affi-Gel 15). Proteins listed in Table 1.

Table 1. Protein Coupling to Affi-Gel 10 and Affi-Gel 15 at pH 7.5.

Protein	pI	Coupling Efficiency, % Affi-Gel 10	Affi-Gel 15
Fetuin	3.3	3.0	43
α-1-antitrypsin	4.0	5.0	76
Ovalbumin	4.7	8.5	70
Bovine serum albumin	4.9	14	80
Human transferrin	5.9	36	87
Bovine hemoglobin	6.8	83	59
Human globulin	5.8–7.3	90	39
Myoglobin	6.8–7.8	85	10
Cytochrome c	9.3	90	0
Lysozyme	10–11	95	1

Affi-Gel 10 couples proteins best at a pH near or below their pI, and Affi-Gel 15 couples proteins best near or above their pI. This is shown for coupling at pH 7.5 in Figure 3 and Table 1. Therefore, when coupling at neutral pH (6.5–7.5), Affi-Gel 10 is recommended for proteins with a pI of 6.5 to 11 (neutral or basic proteins), and Affi-Gel 15 is recommended for proteins with a pI below 6.5 (acidic proteins).

The difference in coupling efficiency of Affi-Gel 10 and Affi-Gel 15 for acidic and basic proteins can be attributed to interactions between the charge of the protein and charge on the gel. Hydrolysis of some of the active esters during aqueous coupling will impart a slight negative charge to Affi-Gel 10. This negative charge will attract positively charged proteins (proteins buffered at a pH below their pI) and enhance their coupling efficiency. Conversely, the negative charge will repel negatively charged proteins (proteins buffered at a pH above their pI) and lower their coupling efficiency. Affi-Gel 15, due to the tertiary amine incorporated into its arm, has a slight overall positive charge, and the effects are reversed.

In addition to its effect on coupling, the slight charge associated with each gel may sometimes be exploited in the affinity separation itself — for example, it may be used to enhance binding of weakly adsorbed materials, or elution of strongly adsorbed materials. In such cases, it may be preferable to use Affi-Gel 10 to couple an acidic protein, or Affi-Gel 15 to couple a basic protein. Coupling efficiency can then be enhanced by manipulating the coupling conditions in either of two ways.

Table 2. Coupling Efficiency of Acidic and Neutral-to-Slightly-Basic Protein under Various Coupling Conditions.

Coupling Buffer	Coupling Efficiency, % Affi-Gel 10	Affi-Gel 15
Bovine serum albumin, pI 4.9		
0.1 M MOPS, pH 7.5	14	80
0.1 M MOPS, pH 7.5 + 80 mM $CaCl_2$	90	—
0.1 M MOPS, pH 7.5 + 0.3 M NaCl	22	47
0.1 M MES, pH 4.8	90	38
Human globulin, pI 7.0 (average)		
0.1 M MOPS, pH 7.5	83	40
0.1 M MOPS, pH 7.5 + 0.3 M NaCl	69	70
0.1 M $NaHCO_3$, pH 8.5	80	70

Select the coupling pH so that the protein has a charge opposite that of the gel, or add salt to the coupling buffer to minimize charge interaction (80 mM $CaCl_2$ is recommended for coupling acidic proteins to Affi-Gel 10, and 0.3 M NaCl is recommended for coupling basic proteins to Affi-Gel 15). Examples of these manipulations are shown in Table 2. The more basic or more acidic the protein, the larger the observed effects will be.

Temperature

Coupling at 4°C is recommended whenever possible. The slower reaction rate at this temperature will afford a greater measure of control. Although the reaction will proceed at a faster rate at 20°C, the reaction efficiency is comparable and many ligands are more stable at 4°C.

Time

Coupling to Affi-Gel 10 and 15 is rapid. As shown in Figure 4, for Affi-Gel 10, about 75% of the maximum binding achieved with γ-globulin at pH 8.0 takes place within 30 min at 4°C. In 1 hr, 80% of the maximal coupling is achieved, and within 4 hr the reaction is complete.

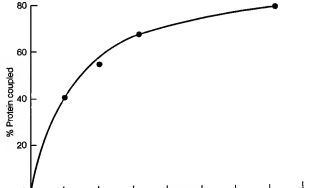

Fig. 4. Effect of time on protein coupling to Affi-Gel 10. A similar relationship is observed with Affi-Gel 15. Human γ-globulin (15 mg/ml of gel) was added in 0.1 M HEPES, pH 8.0 at 4°C.

Ligand Concentration

The amount of protein coupled is proportional to the amount of protein added to the gel, up to about 30 mg coupled/ml of gel (Figure 5). The efficiency of coupling will vary with the protein and conditions of coupling (see Figure 3). Above 30 mg protein/ml gel, more protein may be coupled, but efficiency will taper off. When maximum capacity is desired, a high concentration of ligand should be chosen (50–60 mg protein per ml of gel). When maximum efficiency is the goal, as would be the case with ligand preparation of limited quantity, the ligand concentration should be in the range of 25 mg protein/ml of gel. The sample that remains after coupling may be recovered and reused since it will not be subject to chemical modification by any reaction other than attachment to the gel. The optimum coupling efficiency is achieved when the total reaction volume is between 1.5 and 4.5 ml per ml of gel bed. The optimum ligand concentration for antibody applications is usually 5–10 mg antibody per ml of gel. The minimum concentration is 1 mg/ml.

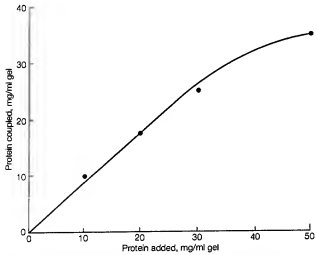

Fig. 5. Effect of amount of protein added on protein coupling to Affi-Gel 10. Human γ-globulin in 0.1 M MOPS, pH 7.5. A similar coupling capacity is observed with Affi-Gel 15.

Recommended Storage Conditions

Store Affi-Gel 10 in a freezer (-20°C) to retain 80% of original activity for at least one year. Store at -70°C to extend shelf life well beyond one year.

General Instructions

Aqueous Coupling

Shake the vial, and observe that a uniform suspension exists in the vial. Transfer the desired quantity of slurry to a small Buchner funnel or glass fritted funnel. Drain the supernatant solvent, and wash the gel with 3 bed volumes of cold (4°C) deionized water. The wash can be facilitated, particularly when working with larger amounts of gel, by applying a vacuum. Care should be taken, however, not to allow the gel bed to go dry. For optimum coupling of ligands, the washing procedure should be completed and the gel combined with the ligand solution within 20 min.

Transfer the moist gel cake to a test tube or flask and add the cold ligand solution. Add at least 0.5 ml of ligand solution/ml of gel, and agitate sufficiently to make a uniform suspension. Continue gentle agitation of the gel slurry on a rocker, shaker, or wheel for 1 hr at room temperature or 4 hr at 4°C.

If the coupling time is short, or if the gel is to be used immediately, we recommend a precautionary blocking of any active esters that might remain. This can be accomplished by adding 0.1 ml of 1 M glycine ethyl ester (pH 8) or 0.1 ml of 1 M ethanolamine-HCl (pH 8) per ml gel. Allow 1 hr for completion of the blocking reaction. Transfer the gel to a column and wash with water or coupling buffer until the gel is free of reactants detected by A_{280}. Wash the gel with other solvents that will be used subsequently to elute substances specifically bound to the column. The column is now ready for equilibration with starting buffer and application of sample. If the column is not to be used immediately, store it at 4°C or with the gel equilibrated in a solution containing 0.2% sodium azide.

Monitoring Protein Coupling

Soluble (unbound) protein remaining in the coupling and wash buffers can be quantitated using the Bio-Rad protein assay (catalog #500-0006) or by measuring its absorbance at 280 nm. If absorbance at 280 nm is preferred, the pH of the sample should be lowered by diluting in 0.01 N HCl. At neutral or basic pH the N-hydroxysuccinimide released during the coupling will absorb at 280 nm. N-hydroxysuccinimide will also interfere with the Lowry protein assay.

Anhydrous Coupling

Coupling under anhydrous conditions is the preferred method when suitable for the ligand. Since active esters are not hydrolyzed in the absence of water, the only reaction will be that of the ligand with the gel. To obtain a quantitatively substituted gel with low molecular weight ligands, it is necessary to add only a slight excess of ligand (about 15 μmol of active ester are available per ml of gel) in the solvent in which the gel is equilibrated. Suitable solvents include alcohols, dimethylsulfoxide, dioxane, acetone, dimethylformamide (grades that are contaminated with free amines should be avoided), or mixtures of these solvents. In the absence of hydrolysis, other factors such as time, concentration, and temperature are less important considerations. The reaction can be carried out in any convenient volume at room temperature for several hours. Any unreacted groups that remain can be blocked by addition of a slight excess of ethanolamine. The resulting support will have the lowest possible residual charge.

Table 3 lists typical applications of Affi-Gel 10, and specific application examples are shown for phosphodiesterase (Figure 6) and for prolactin receptors (Figure 7).

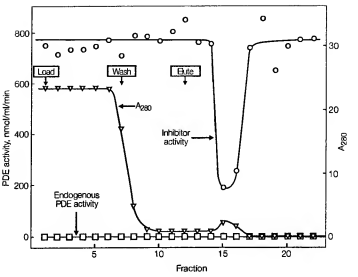

Fig. 6. Activator-agarose affinity column (calmodulin-agarose)* chromatography. Pooled phosphodiesterase (PDE) containing fractions from an Affi-Gel Blue column were adjusted to 1.5 mM $CaCl_2$, mixed with 15 ml of activator-agarose, and stirred gently overnight. The suspension was poured into a column (2.5 x 10 cm), washed with 60 ml of 50 mM Tris-HCl (pH 7.8, 3 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM dithiothreitol, and then eluted. Fractions of 10 ml were collected; an aliquot of each was assayed for inhibitor (○—○) and PDE (□—□) activities. For more information, refer to Wallace et al. (1979, 1980).

* Calmodulin coupled to Affi-Gel 10.

Table 3. Applications of Affi-Gel 10.

Ligand Bound to Affi-Gel 10	Application	Reference
Enzymes		
Chymotrypsin	Isolation of inhibitors	Twining and Brecher 1975
Gentamicin C_{1a}	Purification of acetyltransferase	Williams and Northrup 1976
Plasminogen	Immobilized protease	Chibber et al. 1974
D-tryptophan methyl ester	Purification of α-chymotrypsin	Wilkinson et al. 1976
Lactoperoxidase	Solid-phase radioiodination of CEA	Tsao and Kim 1978
Soybean trypsin inhibitor	Removal of plasmin from plasminogen	Bouma et al. 1980
Immunoadsorption		
Fibrinogen	Sorption on antibodies	Chen and Shurley 1975
Antibody	Isolation of microtubule protein	Ikeda and Steiner 1976
Antibody	Isolation of brush-border aminooligopeptidase	Maze and Grey 1980
p-phenylazotyrosine (arsenic) hapten	Antihapten antibody	Kanellopoulos et al. 1979
Monospecific IgG	Purification of *Chlamydia trachomatis*–specific antigen	Caldwell and Kuo 1977
Anti-CEA antibody	Solid phase radioimmunoelectrophoretic assay of CEA	Saravis et al. 1974
Cell Membrane Receptors		
hGH	Purification of prolactin	Shiu and Friesen 1974
hCG	Purification of hCG receptors	Dufau et al. 1975
Insulin	Purification of human insulin receptors for production of specific antibodies	De Pirro et al. 1979
GnRH	Cellular gonadotropin release	Conn et al. 1981
Other		
Amino acids	General usage	Cuatrecasas and Parikh 1972
Polymyxin	Immobilized growth inhibitors to *E. coli*	La Porte et al. 1977
Castor bean lectin	Affinity chromatography of tyrosine transfer RNA	Garcia and Singhal 1979
Bovine serum albumin	Preparative purification of mouse immune interferon	Osborne et al. 1979
Hemagglutinin neuraminidase	Study of response of cytolytic thymus-dependent lymphocytes	Guertin and Fan 1980
L-tryptophyl-L-tryptophan	Human interferon purification	Zoon et al. 1979
Gelatin	Fibronectin purification	Colter 1980
Calmodulin	Isolation of calmodulin-binding protein	Wallace et al. 1979, 1980

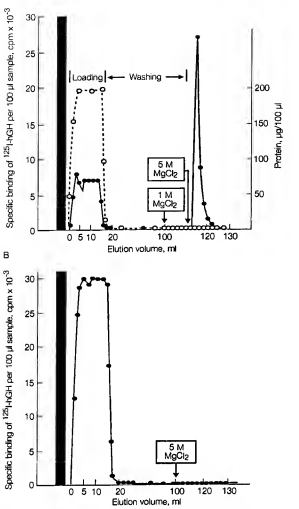

Fig. 7. Purification of prolactin receptors by Affi-Gel 10 chromatography. A 7.5 ml Triton X-100 extract of membrane (2 mg/ml) from mammary tissue was applied to 5 ml columns packed with Affi-Gel 10 coupled to either hGH (A) or BSA (B) (●—●); specific binding; (○—○), amount of protein. Solid bar represents receptor activity in 100 µl of the extract.

Immobilization of Purified Con A and Purification of Horseradish Peroxidase (HRP)

A highly effective method for preparing immobilized concanavalin A (Con A) and for purifying horseradish peroxidase was developed in our laboratories using ovalbumin, jack bean meal, and Affi-Gel 15 (Figure 8).

A solution of ovalbumin (40 mg/ml in 0.1 M HEPES, pH 7.5) was combined with an equal volume of Affi-Gel 15 and mixed at 4°C for 2 hr. Coupling efficiency was 62% (23 mg ovalbumin bound/ml of gel).

Crude jack bean meal was then extracted with phosphate-buffered saline (PBS) and the extract applied to the ovalbumin-agarose gel. After contaminants were washed out with PBS, the bound Con A was eluted with PBS containing 0.3 M α-methylmannoside. Capacity was 10 mg Con A/ml of ovalbumin-agarose.

The purified Con A (10 mg/ml in PBS-α-methylmannoside) was added to an equal volume of Affi-Gel 15 and mixed for 2 hr at 4°C. The coupling efficiency was 91% (9.1 mg of Con A coupled/ml of gel).

Impure HRP, 9 mg (70 U/mg), was applied to 10 ml of Con A-agarose prepared above. After a PBS wash the HRP was eluted with 0.1 M α-methylmannoside in PBS. The specific activity was increased 2.7-fold to 190 U/mg with a recovery of 90% (Figure 9). The RZ number (A_{403}/A_{278}) increased 3.1-fold, from 0.76 to 2.36.

The purified HRP is suitable for conjugation to antibody and the Con A–Affi-Gel 15 column can be used for purification of the conjugate by the method of Arends (1979).

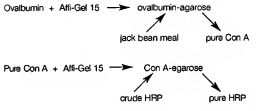

Fig. 8. Overview of HRP purification method.

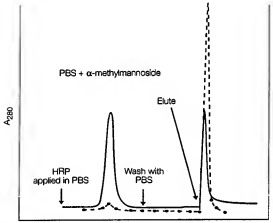

Fig. 9. Purification of horseradish peroxidase (HRP) on Con A–Affi-Gel 15. The solid line represents the absorbance at 280 nm. The broken line (---) represents HRP activity.

Arends J, Purification of peroxidase-conjugated antibody for enzyme immunoassay by affinity chromatography on concanavalin A, J Immunol Methods 25, 171–175 (1979)

Bourne BN et al., Human plasma prekallikrein. Studies of its activation by activated factor XII and of its inactivation by diisopropyl phosphofluoridate, Biochemistry 19, 1151–1160 (1980)

Caldwell HD, and KuomCC, Purification of a *Chlamydia trachomatis*-specific antigen by immunoadsorption with monospecific antibody, J Immunol 118, 437–441 (1977)

Chen JP and Shurley HM, A simple efficient production of neoantigenic antisera against fibrinolytic degradation products: Radioimmunoassay of fragment E, Thromb Res 7, 425–434 (1975)

Chibber BA et al., Immobilized human plasmins: preparation and enzymatic properties, Prep Biochem 4, 315–330 (1974)

Coller BS, Interaction of normal, thrombobasthenic, and Bernard-Soulier platelets with immobilized fibrinogen: defective platelet-fibrinogen interaction in thrombasthenia, Blood 55, 169–178 (1980)

Conn DPM et al., Stimulation of pituitary gonadotropin release does not require internalization of gonadotropin-releasing hormone, J Biol Chem 256, 1098–1100 (1981)

Cuatrecasas P and Parikh I, Adsorbents for affinity chromatography. Use of N-hydroxysuccinimide esters of agarose, Biochemistry 11, 2291–2299 (1972)

De Pirro R et al., Specificities of rabbit anti-human insulin receptor antibodies, J Clin Lab Immunol 2, 27–30 (1979)

Dufau ML et al., Gonadotropin receptors. Solubilization and purification by affinity chromatography, J Biol Chem 250, 4822–4824 (1979)

Garcia CM and Singhal RP, Affinity chromatography of tyrosine transfer RNAs, Biochem Biophys Res Commun 86, 697–703 (1979)

Guertin DP and Fan DP, Stimulation of cytolytic T cells by isolated viral peptides and HN protein coupled to agarose beads, Nature 283, 308–311 (1980)

Ikeda Y and Steiner M, Isolation of platelet microtubule protein by an immunosorptive method, J Biol Chem 251, 6135–6141 (1976)

Kanellopoulos J et al., Préparative isolation of the cell receptor for immunoglobulin E, J Biol Chem 254, 7691–7697 (1979)

La Porte DC et al., Inhibition of *Escherichia coli* growth and respiration by polymyxin B covalently attached to agarose beads, Biochemistry 16, 1642–1648 (1977)

Maze M and Gray G, Intestinal brush border aminooligopeptidases: cytosol precursors of the membrane enzyme, Biochemistry 19, 2351–2358 (1980)

Osborne LC et al., Large-scale production and partial purification of mouse immune interferon, Infect Immun 23, 80–86 (1979)

Saravis CA et al., Solid-phase radioimmunoelectrophoresis: an assay for carcinoembryonic antigen, J Natl Cancer Inst 53, 921–926 (1974)

Shiu RP and Friesen HG, Solubilization and purification of a prolactin receptor from the rabbit mammary gland, J Biol Chem 249, 7902–7911 (1974)

Tsao D and Kim YS, Glycoproteins from human colonic adenocarcinoma. Isolation and characterization of cell surface carcinoembryonic antigen from a cultured tumor cell line, J Biol Chem 253, 2271–2278 (1978)

Twining SS and Brecher AS, Isolation and identification of a1-antitrypsin as a component of normal and malignant human breast and other tissues, Proc Soc Exp Biol Med 150, 98–103 (1975)

Wallace RW et al., Purification and characterization of an inhibitor protein of brain adenylate cyclase and cyclic nucleotide phosphodiesterase, J Biol Chem 254, 377–382 (1979)

Wallace RW et al., High levels of a heat-labile calmodulin-binding protein (CaM-BP80) in bovine neostriatum, Biochemistry 19, 1831–1837 (1980)

Wilkinson TJ et al., Affinity chromatographic separations of chemically modified α-chymotrypsins from α-chymotrypsin, Sep Sci 11, 385 (1976)

Williams JW and Northrup DB, Purification and properties of gentamicin acetyltransferase I, Biochemistry 15, 125–131 (1976)

Zoon KC et al., Purification and partial characterization of human lymphoblast interferon, Proc Natl Acad Sci USA 76, 5601–5605 (1979)

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